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(54) Title: FOOD SPOILAGE DETECTION METHOD

(57) Abstract

Diacetyl, especially resulting from spoilage of chilled red meat, is monitored by exposing an aromatic ortho-diamine at acidic pH to an environment containing, or possibly containing, diacetyl and detecting any change in absorption or reflection of electromagnetic radiation due to the ortho-diamine.

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FOOD SPOILAGE DETECTION METHOD

This invention relates to a method of detecting food (including beverage) spoilage and is particularly, although not 5 exclusively, concerned with the detection of spoilage in chilled foodstuffs, such as red meats, during processing, transportation and/or storage.

The monitoring and control of the condition of fresh foodstuffs, which have been given enhanced, but not indefinite, 10 shelf life by chilling and refrigerated storage, poses a major problem in our health conscious society. Thus the consumer demands fresh food, conveniently packaged, and with all-the-year round availability in shops and supermarkets, while at the same time insisting that there is no danger of spoilage leading to 15 impaired looks or flavour, or, more importantly, health risk, in the purchased foods.

Oxidation of lipids, nucleotide degradation by endogenous enzymes and bacterial growth processes involving the metabolism of a wide range of food constituents including carbohydrates and 20 amino acids can all contribute to losses in sensory qualities of foods and their ultimate rejection by the consumer. The relative importance of these various spoilage processes may vary from product to product, with conditions of transportation and storage, with intended use etc., but the consequences of 25 bacterial growth are commonly an important contributory factor. This is especially the case for chilled-fresh foods.

Determinations of the extent and/or precise nature of spoilage may be required for routine quality assurance, for regulatory purposes or for establishing the cause for any 30 particular condition and hence appropriate remedial action. Sensory and microbiological criteria are traditionally used for these purposes, but both have their limitations. The subjectivity of sensory data poses many problems in routine use, not least because of the difficulties in assigning meaningful 35 criteria for differentiating "acceptable" from "non-acceptable"

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(or "spoiled from non-spoiled") product. While total bacterial numbers often bear some relationship to acceptability, there are still problems in their use. Not all bacteria growing on food necessarily contribute to spoilage, nor is there a consistent 5 relationship between bacterial numbers and spoilage. Thus, high pH meat (dark, firm, dry: DFD), spoils at lower cell densities than normal-pH meat under identical storage conditions whereas, on vacuum-packaged meat, bacterial numbers may reach a maximum and then remain unchanged for significant periods without 10 obvious sensory change.

Measurement of chemical change, whether of microbial or non-microbial origin, offers a possible alternative to microbiological and sensory criteria. For example, an 15 electrochemical method for glucose and enzyme sensors for nucleotide degradation products and for certain amines have already been proposed.

It has been found by Dainty *et al.*, *J. Appl. Bact.*, (1985), 59, 303-309 and *J. Appl. Bact.*, (1989), 66, 281-289 that the volatile compound diacetyl (butane 2,3-dione) is produced during 20 chill storage of red meats with free access to air (through a permeable film overwrap) or in high O₂/CO₂ gas atmospheres, and that diacetyl has a high potential as an index of freshness/acceptability/spoilage. This compound has been detected consistently during storage, and at relatively early 25 stages when sensory changes were restricted to development of staleness rather than obvious spoilage. Evidence indicating that accumulation may also occur in vacuum packs has also been suggested as a measure of the condition of some citrus fruit juices and the sanitary status of the processes used in their 30 manufacture, and as a means of detecting supplementation of aroma constituents to mask organoleptic defects in butter. Diacetyl has also been employed as an additive in the manufacture of margarine and cheese. Additionally it is produced during yeast fermentation and plays a role in the flavouring of 35 beer.

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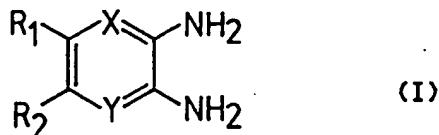
Sensitive, rapid methods for detection and analysis of diacetyl, which can be applied on- or at-line, to monitor food manufacturing processes and quality deterioration during transportation and storage, would therefore be of great value,

5 as such methods would be non-destructive and would minimize the potential hygiene problems associated with the invasive sampling needed for analysis of non-volatile compounds. Such methods have not so far been available.

Accordingly, the present invention provides a method of
10 monitoring the presence of diacetyl comprising exposing an aromatic ortho-diamine at acidic pH to an environment containing, or possibly containing, diacetyl and detecting any change in absorption or reflection of electromagnetic radiation due to the ortho-diamine. The change may suitably, though not
15 necessarily, be in the UV or visible region or both.

The aromatic ortho-diamine preferably has the general formula (I)

20



wherein each X and Y, which may be the same or different, represents -N- or -CH-, R¹ is a conjugated system which may be
25 linear or cyclic or a combination thereof, R² is a conjugated system, which may be linear or cyclic or a combination thereof, or a hydrogen atom or a non-conjugated monovalent substituent group or R¹ and R² together form a cyclic or multicyclic conjugated system; or, when X and/or Y is -CH-, R¹ and X and/or
30 R² and Y together form a cyclic or multicyclic conjugated system.

The diamine may initially be either neutral or in salt form and additionally and preferably, may then have been complexed with a transition metal such as nickel or cobalt. The groups R¹ and R², independently, or when part of a ring system including X or Y, may be substituted. R² may be a hydrogen atom or a simple monovalent substituent such as hydroxy or alkoxy. Preferably R²

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is hydrogen and R¹ is a phenyl or benzoyl group which may be substituted, especially by one or more amino groups.

Especially preferred o-diamines are 3,4-diaminobenzophenone and 3,3'-diaminobenzidine, especially when complexed with a transition metal such as nickel or cobalt in the presence of an inorganic ligand such as chloride or perchlorate. A particularly preferred o-diamine is a complex of nickel chloride with 3,3'-diaminobenzidine which has been found to exhibit a colour change from pale mauve to brown/red, readily detectable with the naked eye.

The invention also includes the transition metal complexes of compounds of formula I, which are believed to be novel *per se* and have been found to have particular utility in the detection of diacetyl.

It will be appreciated that the selection of o-diamine will depend on the use to which the monitoring method is to be put, it being borne in mind that there is a need to protect the foodstuffs from direct contact or contamination with diamines which could be considered as toxic.

The diacetyl, when present, may be in liquid or gaseous phase, but, especially when the method is being used for food monitoring, it will conveniently be in gaseous phase released to the environment adjacent the foodstuff as a result of bacterial changes in the foodstuff. It has been found that, employing certain ortho-diamines as defined above, it is possible to detect visually concentrations of diacetyl in the region of 10 ppm.

The change in absorption or reflection of electromagnetic radiation due to the ortho-diamine may conveniently be a colour change detected visually, especially if only a qualitative monitor of the presence or absence of diacetyl is required. Alternatively, the change may be detected by any convenient spectroscopic method such as measurement of the transmittance and/or reflectance spectra, suitably employing fibre optics. The change may be detected by removing a sample of gas from the environment in which diacetyl may be present for detection, for

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example, spectroscopically. However, preferably, the monitoring is carried out in situ, for example by providing a suitable o-diamine doped substrate on the packaging or adjacent the foodstuff to be monitored, suitably by using a fibre light guide 5 to and from the doped substrate to enable detection by remote measurement. Alternatively an optical fibre may be itself coated with the o-diamine and, if necessary, the coating sealed with a further coating layer of transparent or translucent, diacetyl-permeable, polymeric material. Such an optical fibre 10 may, for example, be mounted within a storage refrigerator to enable detection in situ of a colour change resulting from spoilage of stored foodstuffs. It is envisaged therefore that optical fibres, or a network thereof, may be used in connection with commercial refrigeration plants, the fibres acting to allow 15 monitoring of the transmission of thin film of suitably activated diamine, the reflectance of a thin film of suitably activated diamine or the transmission characteristics of a fibre cladded with a thin film of suitably activated diamine.

Therefore, according to a further aspect of the invention, 20 there is provided an apparatus suitable for use in the monitoring method as described above comprising a source of electromagnetic radiation of wavelength covering the region in which the change is to be detected, means for transmitting such radiation to an aromatic ortho-diamine, means for exposing the 25 ortho-diamine to the environment to be monitored and means for detecting a change in absorption or reflection of the electromagnetic radiation due to the ortho-diamine.

The invention is intended to be applicable to such activities as monitoring the spoilage of chilled red meats with 30 free access to air, or in high O₂/CO₂ atmospheres, in vacuum packs, for example in packs of certain citrus fruit products such as juices or in monitoring the spoilage or change in condition of products including butter substitutes and beer. The invention is also applicable to the monitoring of the "gaminess" 35 of hung game, for example, with the aim of assessing the precise day on which the product has reached premium condition.

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It will be appreciated that the monitoring method of the invention can be used to detect spoilage or change in food condition, during initial processing and packaging, during transportation, for example from one country to another in chilled containers, or during more local distribution to the retailer, as well as at the retail outlets themselves. A preferred method of carrying out the invention therefore comprises the incorporation of the aromatic ortho-diamine into the package or container for those foodstuffs which are known to generate diacetyl, so that any diacetyl generated can be monitored at one or more of the various stages of processing, packaging, transportation, distribution and retailing.

Therefore according to a further aspect of the invention, there is provided a foodstuff package, at least a portion of which incorporates an aromatic ortho-diamine, the diamine being positioned so as to be in contact with the environment surrounding the foodstuff.

The invention also provides a device intended for handling foodstuff known on spoilage or change in condition to generate diacetyl, at least a portion of which device incorporates an aromatic ortho-diamine, the diamine being positioned so that, in use, it will be in contact with the environment surrounding the foodstuff. The device can conveniently be a container for the transportation of such foodstuff, but may also be part of a processing or packaging line for the foodstuff. The term foodstuff is intended to include beverages such as fruit juice and beer.

The ortho-diamine may conveniently be applied to such a package or device in the form of a patch bearing the ortho-diamine, integral with the packaging or container, or subsequently applied thereto. Such a patch may be applied initially at the packaging station or at a late stage. It may be convenient to apply a series of such patches to monitor different stages in the progress of the foodstuff from source to retailer. Any acid component so as to give an acidic pH for the

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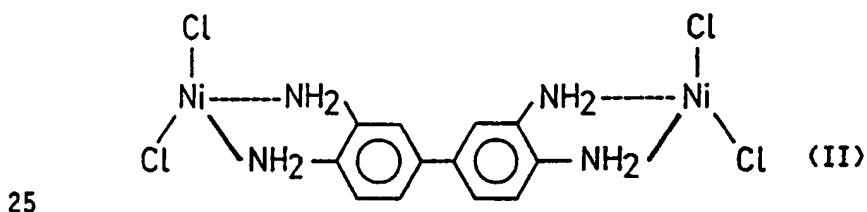
detection of diacetyl using the ortho-diamine can be provided at source or at a later stage when it is desired to effect monitoring. It will be appreciated that such a patch, while being in contact with the environment containing the foodstuff, 5 need not contact the foodstuff itself, but can be in a semi permeable region of the package. The diamine may be protected by an exterior coating from external damage so long as the change in absorption or reflection can still occur and be detected. The use of patches as described above is particularly convenient 10 when the ortho-diamine is capable of giving a visually-detectable colour change on exposure to diacetyl. When monitoring a visually-detectable change, by reflectance, the colour change can be enhanced by incorporating secondary colouring material into the activated diamine.

15 The invention will now be further described by way of example.

Example 1. Preparation of o-Diamine/Transition Metal Complexes

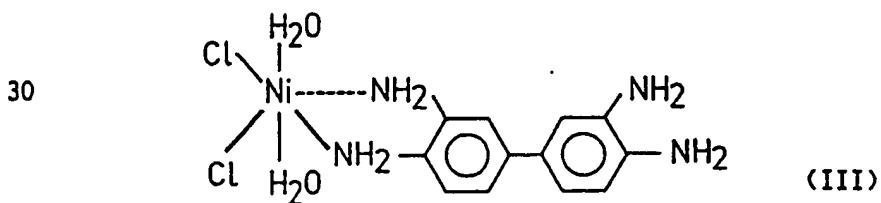
a) 3,3'-diaminobenzidine/nickel dichloride complexes (II) and (III)

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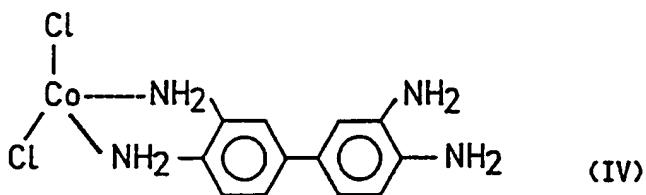
35 3,3'-diaminobenzidine (0.001M; 0.23g) in ethanol (25ml) was added dropwise under reflux to $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, (0.002M; 0.47g) in ethanol (5ml). After 3 hours the reaction mixture was cooled in

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air, a pale dirty white precipitate was collected by vacuum filtration, which was washed with ethyl acetate, petroleum ether and water, then dried in an evacuated dessicator over P_2O_5 . The addition of water changed the complex's colour to pale mauve. Despite a 2:1 addition of $NiCl_2 \cdot 6H_2O$ to diamine, the octahedral 1:1 complex (III) was always formed in the presence of water. In the absence of water a tetrahedral, 2 metals to 1 ligand, complex (II) was obtained. The reflux time was varied and longer times gave the same results as the product after 2-3 hours. Larger quantities in the same ratios yielded the same product.

b) 3,3'-diaminobenzidine/cobalt dichloride complex (IV)

15



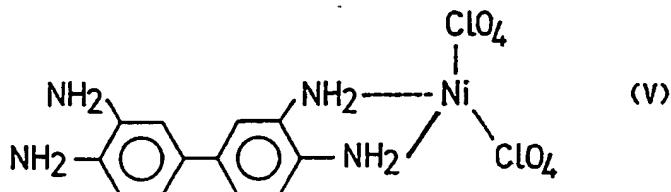
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The reaction described in a) above was carried out except that $CoCl_2 \cdot 6H_2O$ in ethanol was used instead of $NiCl_2$. On cooling, the precipitate was filtered under vacuum, washed with ethanol and dried in an evacuated dessicator over P_2O_5 .

25

c) 3,3'-diaminobenzidine/nickel dichlorate complex (V)

30



35

$Ni(ClO_4)_2 \cdot 6H_2O$, (0.004M; 1.6g), in ethanol was heated under reflux, 3,3'-diaminobenzidine was added (0.002M) dropwise in

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ethanol. This was refluxed for 5 hours. The pale grey product was washed with warm ethanol, then warm petroleum spirit (40-60°C) and finally warm methyl acetate (approx. 50ml each).

The resulting solid was dried as described in a) above.

5 The above reaction was repeated but using chloroform instead of ethanol. The reaction mixture (using the same ratios of reactants as given above) was refluxed for 6 hours and then left to cool for 12 hours before refluxing for a further 8 hours. The resulting pale grey solid was collected by vacuum 10 filtration, washed with chloroform and dried as described in a) above.

Example 2. Use of o-Diamines and their Transition Metal Complexes for Detection of Diacetyl

A dilute ethanolic solution of each of II, III, IV and V was 15 prepared, together with similar solutions of 3,3'-diaminobenzidine and 3,4-diaminobenzophenone. Compound VI was made up in dimethylformamide.

One or two drops of concentrated hydrochloric acid were added for each approximately 50ml of solution. A strip of 20 Whatman filter paper was soaked in this solution and then air dried. The strip was now ready to be exposed to diacetyl. This was done in an enclosed flask containing a low concentration (minimum 10 ppm approx.) of diacetyl. All the dyes and 25 complexed dyes reacted with diacetyl resulting in a colour change observable with the naked eye. However, the attendant changes in the UV region were up to 100 times more intense than those in the visible region.

Examples of the colour changes which were obtained are as follows:

30	<u>Dye</u>	<u>Metal Salt</u>	<u>Colour Change</u>
	3,3'-diaminobenzidine	nickel chloride hexahydrate (III)	grey→yellow/red
35	3,3'-diaminobenzidine	cobalt chloride hexahydrate (IV)	blue→orange/yellow

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	3,3'-diaminobenzidine	nickel chloride hexahydrate (V)	pale purple→orange/ yellow
5	3,4'-diaminobenzo- phenone	nickel chloride hexahydrate	pale green→yellow

Other dyes, when evaporated onto filter paper, were colourless, but on exposure to diacetyl vapour, developed a 10 yellow colouration.

It was found that with compound (III) approximate diacetyl vapour concentrations as low as 0.6 ppm produced an observable colour change. Clearly, the use of intensity changes in the UV enable levels of diacetyl below 0.1 ppm to be detected.

15 Figures 1, 2 and 3 show transmission measurements of the exposed dye (i.e. to diacetyl) relative to an unexposed dye reference film for compounds III, IV and V respectively. It will be appreciated that all spectroscopic features shown are solely due to reaction between the dye and the diacetyl.

20 Example 3. Use of o-Diamines in Detecting Diacetyl

A series of eight commercially available o-diamines were tested for their ability to detect diacetyl as follows:

25 Test-tube experiments were set up using microspatula quantities of the diamine and equal quantities of solvent, diacetyl and concentrated hydrochloric acid. The solvent used depended on the solubility of the diamine. Controls of diamine,

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acid and solvent as well as diacetyl, acid and solvent were also set up for comparison with the reaction test-tubes. The results obtained were as follows:

	<u>Diamine</u>	<u>Solvent</u>	Colour without <u>diacetyl</u>	Colour with <u>diacetyl</u>
5				
10	3,4-diaminobenzoic acid 4-chloro-1,2-phenylene- diamine		pale orange pale pink	khaki green khaki green
	3,3'-diaminobenzidine		deep orange	dark brown
	3,4-diaminobenzophenone		pale orange	lime green
	2,3-diaminonaphthalene		pale golden	dark green
15	4,5-dimethyl-1,2- phenylene-diamine		dark orange	dark brown & fawn precipitate
	1,2-phenylene diamine		pale pink	lime green
	2,3-diaminotoluene		deep orange	dark brown
20	Most of these reactions were not immediate but happened over a period of time. The longer the solutions were left the more the colour developed.			

The reaction conditions were investigated as follows:

25 Variable concentrations of acid from 2 molar to concentrated were used with quantities of diacetyl and diamine. The diamines used were those which had previously shown a positive colour reaction. It was found that the reaction would only proceed with concentrated acid, but only a quarter of the amount of acid to diacetyl/diamine was needed to implement the reaction.

30 Diamine concentration was effectively reduced to 0.002 molar concentration, dilution being with either ethanol or methyl sulfoxide.

35 Reactions were carried out at room temperature, but on heating the reaction proceeded more rapidly and on cooling in ice the reaction proceeded more slowly.

40 Various dilutions of diacetyl in the appropriate solvent were tested with the already established optimum conditions. Significant colour changes were still observed using 0.08% concentrations of diacetyl.

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To investigate the use of diacetyl vapour, a simple experiment was set up using concentrated diacetyl and stoppered boiling tubes. Strips of filter paper were dipped into the appropriate diamine solutions and allowed to dry. They were 5 then inserted into the bungs which were used to stopper boiling tubes containing small amounts of diacetyl. The tubes were left to stand to allow a build-up of diacetyl vapour within them and the filter papers compared with controls with no dried diamine on them. The colour changes on the filter papers compared 10 favourably with those seen in solution.

Using optimum conditions already established 0.0001 molar concentration transition metal halides were introduced into the reaction mixtures. Colour changes were very strong for those 15 solutions in which the reaction was already pronounced. The colour changes were cobalt (blue to green), magnesium (green to yellow), iron (green to colourless).

Example 4. Use of Fibre Optics in Detecting Diacetyl

Diacetyl is detected using a flow rig as shown schematically in Figure 4. The rig comprises a light source covering the 20 spectral range of interest. For the compounds described above which exhibit colour change, this will be the visible region. Preferably, the range is versatile so that the most intense spectral change for each dye used can be investigated with the same light source.

25 The modulating device is a mechanical device which chops the incoming light at a particular frequency, while the mono-chromator, with a stepper motor, allows scanning of a spectral range so that the peak wavelength at which a change in colour is most intense for a particular dye is found. The fibre optics 30 comprises a fibre bundle which uses the fibres as a light guide to and from the sample, i.e. a suitable base material coated with a dye as described above. The sample can be positioned at a site remote from the light source and detection equipment, which site is in contact with, or can be brought into contact

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with, the environment being monitored for diacetyl content. Such an environment will for example include the foodstuff or beverage which may be of unacceptable freshness. It will be appreciated that the light source and other equipment can 5 therefore be protected from the potentially cold damp atmosphere where the foodstuff or beverage is being stored, e.g. a refrigerator.

The detection device is a photodiode which produces a readable current from the intensity of the incoming light while 10 the amplifier amplifies the current output from the photodiode and converts the reading to a voltage output. The lock-in amplifier is directly connected to the mechanical chopper and locks in to that frequency, which means that only light which is at the chopper's frequency is read, so that ambient light is 15 excluded. The analogue reading passes to the computer terminal which can perform operations such as recording, storing and manipulation of the measurements.

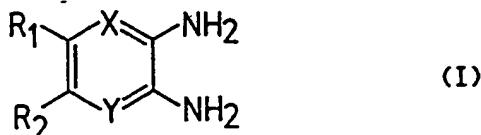
It will be appreciated that the detection means and energising means can be varied to suit the particular 20 application.

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CLAIMS

1. A method of monitoring the presence of diacetyl comprising exposing an aromatic ortho-diamine at acidic pH to an environment containing, or possibly containing, diacetyl and
- 5 detecting any change in absorption or reflection of electromagnetic radiation due to the ortho-diamine.
2. A method according to claim 1 wherein the aromatic ortho-diamine has the general formula (I)

10



- wherein each X and Y, which may be the same or different,
- 15 represents -N- or -CH-, R¹ is a conjugated system which may be linear or cyclic or a combination thereof, R² is a conjugated system, which may be linear or cyclic or a combination thereof, or a hydrogen atom or a non-conjugated monovalent substituent group or R¹ and R² together form a cyclic or multicyclic conjugated system; or, when X and/or Y is -CH-, R¹ and X and/or R² and Y together form a cyclic or multicyclic conjugated system.
- 20 3. A method according to claim 2 wherein R² is hydrogen and R¹ is a phenyl or benzoyl group, which may be substituted.
4. A method according to claim 3 wherein the ortho-diamine is
- 25 3,4-diaminobenzophenone or 3,3'-diaminobenzidine.
5. A method according to any one of the preceding claims wherein the ortho-diamine is present as a transition metal complex.
6. A method according to claim 5 wherein the ortho-diamine is a
- 30 complex of nickel chloride with 3,3'-diaminobenzidine.
7. A method according to any one of the preceding claims intended for the monitoring of diacetyl produced during spoilage or change in condition of fresh foodstuffs.
8. A method according to claim 7 intended for the monitoring of
- 35 diacetyl produced during spoilage of red meat.

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9. A method according to any one of the preceding claims wherein the detection of change in absorption or reflection of electromagnetic radiation due to the orthodiamine is carried out using fibre optics.
- 5 10. Transition metal complexes of compounds of formula I as defined in claim 2.
11. A nickel or cobalt transition metal complex of 3,4-diaminobenzophenone or 3,3'-diaminobenzidine.
12. A complex of nickel chloride with 3,3'-diaminobenzidine.
- 10 13. An apparatus, suitable for use in the monitoring method as defined in any one of claims 1 to 9, comprising a source of electromagnetic radiation of wavelength covering the region in which the change is to be detected, means for transmitting such radiation to an aromatic ortho-diamine, means for exposing the
- 15 14. An apparatus according to claim 13, wherein the means for detecting a change in absorption or reflection of the electromagnetic radiation due to the ortho-diamine.
16. An apparatus according to claim 13, wherein the means for transmitting radiation comprises one or more optical fibres.
- 20 15. A foodstuff package, at least a portion of which incorporates an aromatic ortho-diamine, the diamine being positioned so as to be in contact with the environment surrounding the foodstuff.
16. A device intended for handling foodstuff known on spoilage or change in condition to generate diacetyl, at least a portion of which device incorporates an aromatic ortho-diamine, the diamine being positioned so that, in use, it will be in contact with the environment surrounding the foodstuff.
- 25 17. A foodstuff package according to claim 15 or a device according to claim 16 wherein the diamine is borne by a patch forming part of or applied to the package or device.
18. A foodstuff package or device according to any one of claims 15 to 17 wherein the diamine is as defined in any one of claims 2 to 6.
- 30 35 19. A foodstuff package or device according to any one of claims 15 to 18 wherein the foodstuff is selected from red meat, game and citrus fruit products.

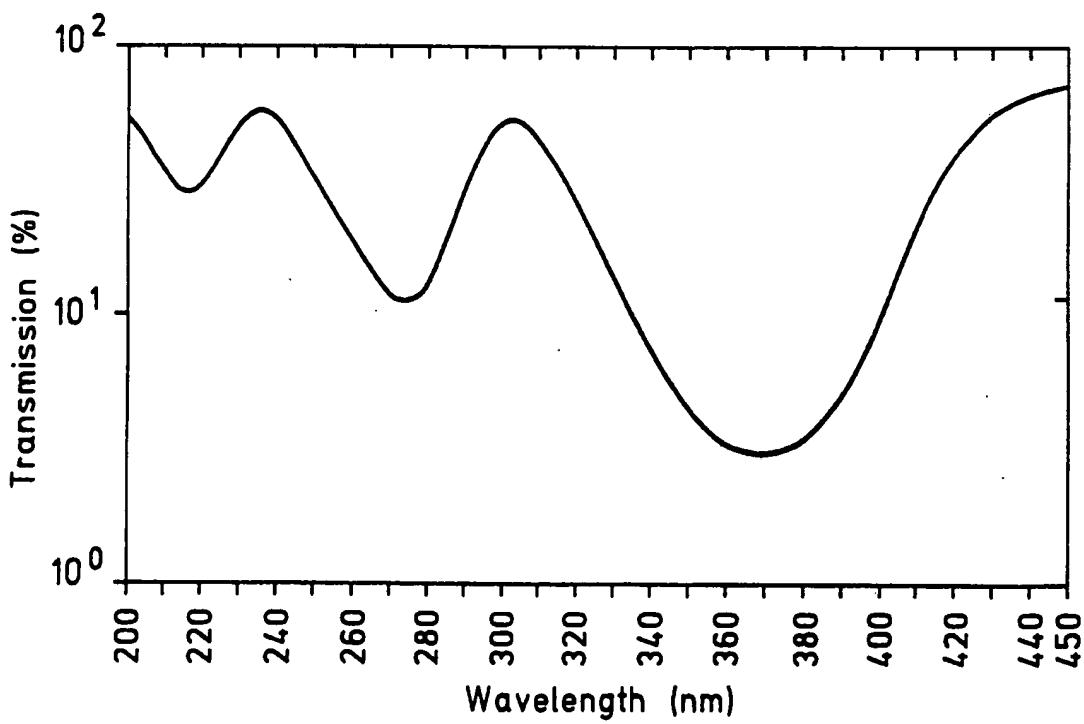
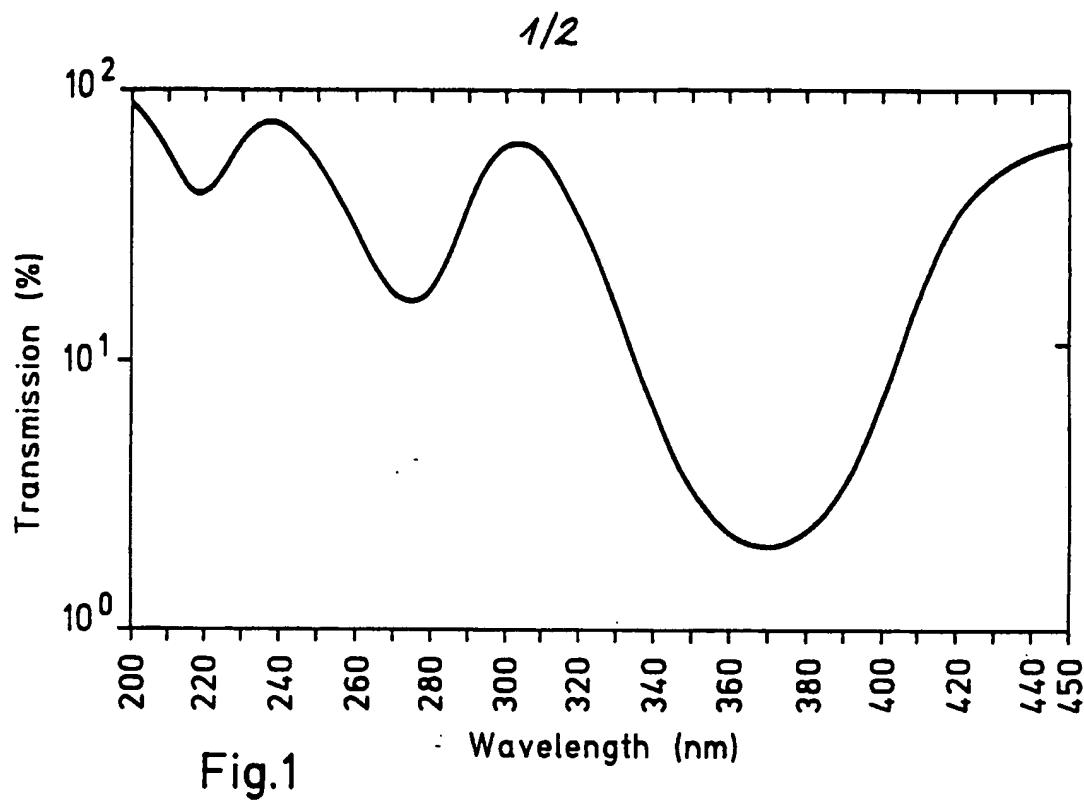


Fig.2

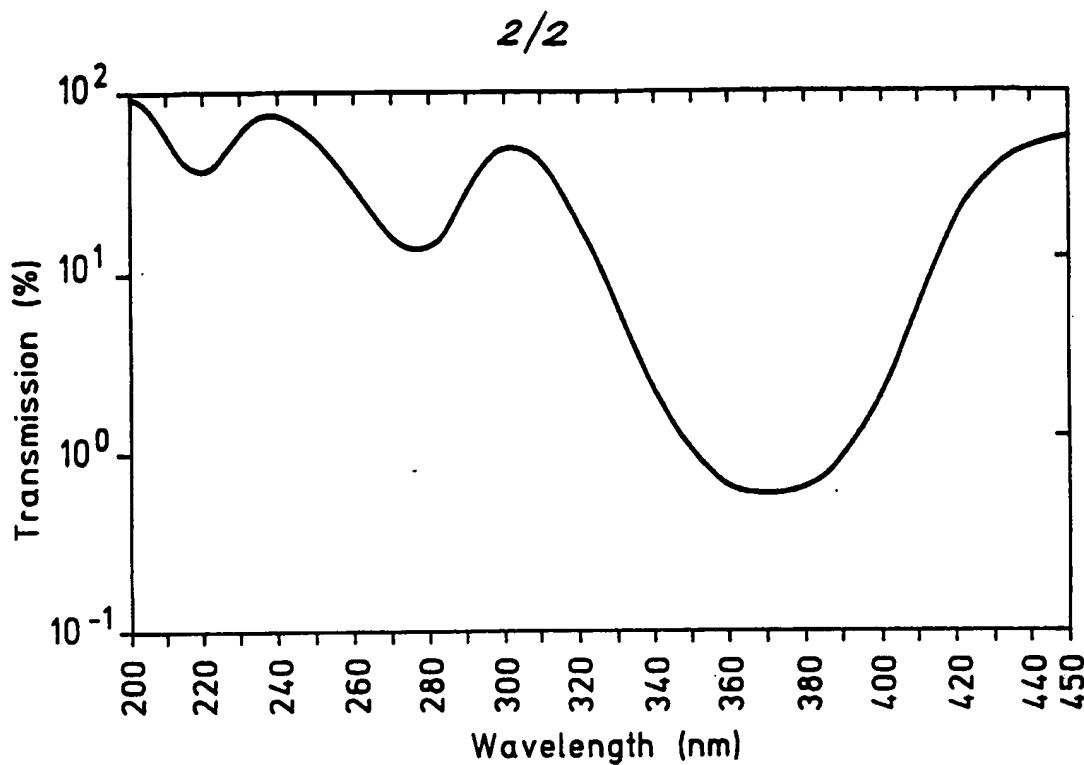


Fig.3

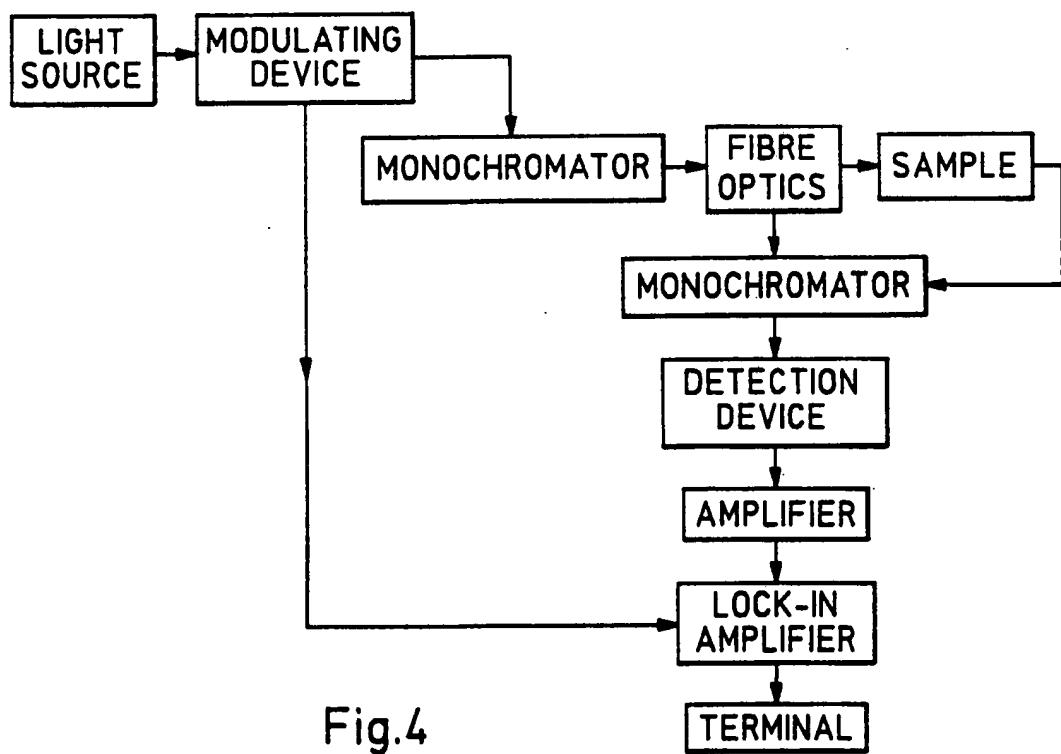


Fig.4

INTERNATIONAL SEARCH REPORT

PCT/GB 93/00155

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 G01N33/12; G01N21/31; C07F15/04; C07F15/06
G01N33/02; G01N31/22

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.C1. 5	G01N ; C07F

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	GB,A,2 013 336 (S.A. BRASSERIES KRONENBOURG) 8 August 1979 see the whole document ----	1-13
A	JOURNAL OF FOOD SCIENCE. vol. 55, no. 4, July 1990, CHICAGO US pages 1164 - 1165 G.SADLER, M.PARISH, AND J.DAVIS 'Diacetyl Measurement in Orange Juice Using Differential Pulse Polarography' see the whole document ----	1,7,8, 13,14 -/-

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- ^P document published prior to the international filing date but later than the priority date claimed

- ^T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- ^X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- ^Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- ^A document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

28 MAY 1993

Date of Mailing of this International Search Report

17.06.93

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

DÖPFER K.P.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>CHEMICAL ABSTRACTS, vol. 110, no. 13, 27 March 1989, Columbus, Ohio, US; abstract no. 113300, GARCIA ESTEPA, R.M. ET AL. 'Spectrophotometric determination of alpha-diketones in citrus juices by formation of diisonicotine hydrazone-zirconium(IV) complexes' page 565 ; see abstract & CIENC. IND. FARM. 1988, 7(10), 281-284 ---</p>	1-13
A	<p>CHEMICAL ABSTRACTS, vol. 113, no. 21, 19 November 1990, Columbus, Ohio, US; abstract no. 191592, CHOO, PEK LEE ET AL. 'A new type of organocobalt complex with a buckled equatorial ligand' page 747 ; see abstract & INORG. CHIM. ACTA 1990, 171(2), 183-192 ---</p>	1,5, 10-12
A	<p>DATABASE WPI Section Ch, Week 8038, 29 October 1980 Derwent Publications Ltd., London, GB; Class G, Page 7, AN 80-66971C/38 & JP,A,55 104 272 (MITSUI TOATSU CHEM. INC.) 7 February 1979 see abstract ---</p>	1-4,7-9
A	<p>CHEMICAL ABSTRACTS, vol. 103, no. 15, 14 October 1985, Columbus, Ohio, US; abstract no. 119176, DUENAS, J.L. ET AL. 'Specific nuclear staining of leukocytes, macrophages and mast cells using a diaminobenzidine-cobalt chloride mixture' page 356 ; see abstract & CELL. MOL. BIOL. 1984, 30(6), 525-528 ---</p>	1-6, 10-12
A	<p>US,A,4 285 697 (MICHAEL P. NEARY) 25 August 1981 see the whole document ---</p>	15-19
		-/-

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>THE JOURNAL OF APPLIED BACTERIOLOGY vol. 59, no. 4, October 1985, OXFORD, GB pages 303 - 309</p> <p>R.H. DAINTY ET AL. 'Time course of volatile compound formation during refrigerated storage of naturally contaminated beef in air' cited in the application</p> <p>---</p>	
A	<p>JOURNAL OF APPLIED BACTERIOLOGY vol. 66, no. 4, April 1990, OXFORD, GB pages 281 - 289</p> <p>R.H. DAINTY ET AL. 'Volatile compounds associated with microbial growth on normal and high pH beef stored at chill temperatures' cited in the application</p> <p>-----</p>	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9300155
SA 69448

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 28/05/93

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		BE-A-	872349	16-03-79
		CA-A-	1122443	27-04-82
		CH-A-	633111	15-11-82
		DE-A-	2852467	02-08-79
		JP-A-	54113396	04-09-79
		LU-A-	80524	22-03-79
		NL-A-	7811390	01-08-79
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